



**UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460**

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

DATE: March 29, 2011

SUBJECT: *HeiQ AGS-20 (Nanosilver Composite)*: Data Evaluation Record (DER) of a Published Literature Study: 28-Day Oral Repeated-dose Study in Mice in Support of New Registration

PC Code(s): Not assigned yet	DP Barcode(s)/No(s): D386911
Decision No.: N/A	Registration No(s): 85249-R
Petition No(s): N/A	Regulatory Action: Data Evaluation Record
Risk Assess Type: Single Chemicals	Case No(s): N/A
TXR No.: 1003206	CAS No(s): Not assigned yet
MRID No(s): 48385201	40 CFR: None

FROM: Jenny Tao, Toxicologist *Jenny Tao*
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THRU: Nader Elkassabany, PhD, Branch Chief *Nader Elkassabany*
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TO: Demon Fuller, Chemical Review Manager
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*Rec'd in RRE
5/6/2011
21*

CITATION: Park, E., E. Bae, J. Yi, et al. (2010) Repeated-dose toxicity and inflammatory responses in mice by oral administration of silver nanoparticles. *Environmental Toxicology and Pharmacology*. 30:162-168. MRID 48385201.

EXECUTIVE SUMMARY: In a repeated-dose oral toxicity study, silver nanoparticles of 42 nm diameters were orally administered in mice, ICR strain, 6 per group at dose levels of 0.25, 0.5, and 1.0 mg/kg/day for 28 days.

At the dose level of 1.0 mg/kg/day, significant increases in serum enzyme level of alkaline phosphatase ($p < 0.01$) and aspartate transaminase ($p < 0.05$) were observed in both males and females. The females group displayed a significant increase in alanine transaminase level ($p < 0.01$). Such increased in serum enzyme levels indicated the liver to be one of the target organs of concern. In addition, a dose-dependent, statistically-significant increase in the concentration of pro-inflammatory cytokines (IL-1, IL-6), Th1-type cytokines (IL-12), Th2-type cytokines (IL-4, IL-10), and TGF- β in serum, and IgE was observed.

A slightly increase in the proportion of B cells was observed in all treatment groups. At the 1.0 mg/kg/day dose level, NK, NKT, B, and T cell distribution were all increased compared to the control group. The ratio of CD4+/CD8+ T cells was decreased indicating an increase of CD8+ T cell distribution.

The changes in clinical chemistry are summarized in Table 1 below.

Table 1. Comparison of Clinical Chemistry and Inflammatory Responses at Dose Level of 1.0 mg/kg/day Treatment Group with Controls in a 28-Day Repeated-dose Study			
	Levels (units)	At dose level of 1.0 mg/kg/day	Control
Males	AST (IU/l)	157.33 \pm 47.98*	79.00 \pm 14.18
	ALT (IU/l)	103.67 \pm 77.31	48.00 \pm 4.58
	ALP (IU/l)	124.33 \pm 37.98**	80.67 \pm 3.79
Females	AST (IU/l)	211.67 \pm 57.18**	79.67 \pm 5.69
	ALT (IU/l)	150.67 \pm 13.05**	36.33 \pm 5.13
	ALP (IU/l)	181.00 \pm 51.51**	87.67 \pm 23.46
Pro-inflammatory cytokines	IL-1 (pg/ml)	8.8 \pm 07.0**	ND ^a
	IL-6 (pg/ml)	13.75 \pm 0.57**	1.44
	TNF- α (pg/ml)	3.41 \pm 0.06	1.21
Th-1 type cytokines	IL-12 (pg/ml)	76.86 \pm 5.20**	35.5
	IFN- γ (pg/ml)	0.52 \pm 0.04	ND
Th-2 type cytokines	IL-4 (pg/ml)	2.7 \pm 0.07*	ND
	IL-5 (pg/ml)	1.34 \pm 0.01	ND
	IL-10 (pg/ml)	29.02 \pm 1.70**	ND
TGF-β	(pg/ml)	6.73 \pm 0.52**	ND
IgE	(ng/ml)	6.04 \pm 0.74*	3.28

^a Not detected.

Significantly different from control group: * $p < 0.05$; ** $p < 0.01$ ($n=3$, mean \pm SD).

A slight cell infiltration in the cortex of the kidneys was observed at 1.0 mg/kg/day dose level in both males and females.

A NOAEL = 0.5 mg/kg/day and LOAEL = 1.0 mg/kg/day were determined for the 28-day repeated-dose oral toxicity study in mice with silver nanoparticles of 42 nm diameter, based on statistically significant changes in clinical chemistry, including increase in serum liver enzymes in males (alkaline phosphatase and aspartate transaminase) and females (alkaline phosphatase, aspartate transaminase, and alanine transaminase).

The 28-day repeated dose oral toxicity study in mice is classified as **unacceptable-upgradeable/guideline** and does not satisfy the Guideline requirements (OPPTS 870.3050; OECD 407) for a 28-day oral toxicity study in rodents. The study is unacceptable but upgradeable if information on the purity of silver nanoparticles, data on statistical significance on cytokine production, B-cell distribution, inflammatory cell infiltrates are provided. Also, reasoning for observing an increase in the CD8+ cell distribution while analyzing the CD4+/CD8+ ratio must be adequately addressed. Others, including food and water consumptions, neurological evaluations, hematology and ophthalmological examinations, and clinical biochemistry determinations along with a detailed gross necropsy, are also required.

Along with the 28-day oral repeated-dose toxicity study, a 14-day oral toxicity study with various sizes (22 nm, 42 nm, 71 nm, and 323 nm) of silver nanoparticles was conducted at a single dose level of 1.0 mg/kg/day in mice (5/group). Statistically significant ($p < 0.01$) amounts of silver nanoparticles were accumulated in all the tested organs (brain, lungs, liver, kidneys, and testes) in mice being administered orally with 22 nm, 42 nm, and 71 nm silver nanoparticles, with the greatest accumulation seen in the 22 nm treatment group. This greatest accumulation at 22 nm diameter size of silver nanoparticles raises significant concerns about penetration, retention, assimilation, and elimination within the organs of silver nanoparticles at smaller size compared to larger size, such as 323 nm. The results are summarized in Table 2 below.

In addition, the level of TGF- β was significantly increased ($p < 0.01$) in mice treated with smaller-sized nanosilver particles, such as 22 nm, 42 nm and 71 nm, but not with larger-sized nanosilver particles (e.g., 323 nm), when compared with the control group.

Table 2. Accumulation of Silver in the Organs in a 14-day Oral Study of Various Sized Nanosilver Particles in Mice at a Single Dose of 1.0 mg/kg/day

Organs	22 nm	42 nm	71 nm	323 nm	Control
Brain	255.56 \pm 3.13**	138.80 \pm 1.85**	51.32 \pm 3.56**	ND ^a	ND
Lung	208.11 \pm 7.65**	171.11 \pm 9.05**	13.00 \pm 22.50	ND	ND
Liver	265.41 \pm 1.55**	86.67 \pm 150.11	220.95 \pm 7.48**	ND	ND
Kidney	345.76 \pm 10.75**	47.92 \pm 16.40**	53.21 \pm 1.53**	ND	ND
Testis	316.00 \pm 5.40**	172.63 \pm 8.75**	ND	ND	ND

^a Not detected.

Significantly different from control group: ** $p < 0.01$ ($n=5$, mean \pm SD).

The Data Evaluation Record (DER) is attached.

Sign-off Date : 03/29/11

DP Barcode No. : D386911

TXR No. : 1,003,206

28-Day Oral Toxicity in (Mice) (2010) / Page 1 of 14
OPPTS 870.3050 / OECD 407

Silver nanoparticles/ (PC Code Not Provided)

EPA Reviewer: Jenny Tao
 Risk Assessment and Science Support Branch
 Antimicrobial Division

Signature: [Signature]
 Date 3-28-11

EPA Secondary Reviewer: Jonathan Chen
 Risk Assessment and Science Support Branch
 Antimicrobial Division

Signature: [Signature]
 Date 03/29/2011

EPA Secondary Reviewer: Melba Morrow
 Risk Assessment and Science Support Branch
 Antimicrobial Division

Signature: [Signature]
 Date 3/29/2011

DATA EVALATION RECORD

STUDY TYPE: Repeated-Dose (28-Day) Oral Toxicity [diet] – mice; OPPTS 870.3050; OECD 407.

PC CODE: Not assigned yet
TXR NO.: 1003206

DP BARCODE: D386911
SUBMISSION NO.: 874753

TEST MATERIAL (PURITY): Silver nanoparticles

SYNONYMS: Nanosilver particles

CITATION: Park, E., E. Bae, J. Yi, et al. (2010) Repeated-dose toxicity and inflammatory responses in mice by oral administration of silver nanoparticles. Environmental Toxicology and Pharmacology. 30:162-168. MRID 48385201.

SPONSOR: Not applicable

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Silver nanoparticles/ (PC Code Not Provided)

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Table 1. Comparison of Clinical Chemistry and Inflammatory Responses at Dose Level of 1.0 mg/kg/day Treatment Group with Controls in a 28-Day Repeated-dose Study			
	Levels (units)	At dose level of 1.0 mg/kg/day	Control
Males	AST (IU/l)	157.33 ± 47.98*	79.00 ± 14.18
	ALT (IU/l)	103.67 ± 77.31	48.00 ± 4.58
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Significantly different from control group: ** $p < 0.01$ (n=5, mean \pm SD).

COMPLIANCE: Signed and dated GLP, Quality Assurance, and No Data Confidentiality statements are not required.

Silver nanoparticles/ (PC Code Not Provided)

I. MATERIALS AND METHODS**A. MATERIALS:****1. Test Material:** Silver nanoparticles (Cat. No. 484059, Sigma-Aldrich, USA)

Description:
 Lot/Batch #:
 Purity:
 Compound Stability:
 CAS #:

2. Vehicle and/or positive control: Tetrahydrofurane, Fluka, 99.8% pure**3. Test animals:**

Species:	Mice	
Strain:	ICR	
Age/weight at study initiation:	6-weeks	
Source:	Orient-Bio Animal Company (Seongnam, Gyunggi-do, Korea)	
Housing:	Not provided. The author stated using the principles from "Guide for the Care and Use of Laboratory Animals issued by the Animals Care and Committee of National Veterinary Research and Quarantine Service" for caring of the animals.	
Diet:	Not provided. The author stated using the principles from "Guide for the Care and Use of Laboratory Animals issued by the Animals Care and Committee of National Veterinary Research and Quarantine Service" for caring of the animals.	
Water:	Not provided. The author stated using the principles from "Guide for the Care and Use of Laboratory Animals issued by the Animals Care and Committee of National Veterinary Research and Quarantine Service" for caring of the animals.	
Environmental conditions:	Temperature: 23 ± 1°C Humidity: 55 ± 5% Air changes: Not provided. Photoperiod: 12 hrs dark/ light cycle	
Acclimation period:	1-week prior to the initiation of the study	

B. STUDY DESIGN:**1. In life dates** – Start: not applicable End: not applicable

2. Animal assignment: Animals were randomly assigned to both 14-day and 28-day for repeated-dose toxicity test to silver nanoparticles. In the 14-day study, various sizes such as 22 nm, 42 nm, 71 nm, and 323 nm of silver nanoparticles were tested at one single dose of 1.0 mg/kg/day with 5 mice per group. In 28-day repeated-dose toxicity test, only one size, 42 nm, silver nanoparticles at various doses of 0.25 mg/kg/day, 0.5 mg/kg/day, and 1.0 mg/kg/day were tested with 6 mice per group. The control group was treated with de-ionized water.

3. Dose selection rationale: No rationale was provided for studying the various silver nanoparticle sizes in the 14-day study or the dose selection rationale with 42 nm diameter size of silver nanoparticles in the 28-day study.

4. Treatment preparation, administration, and analysis: Silver nanoparticles were suspended with sonication with Tetrahydrofurane, Fluka, which was evaporated by stirring the suspension. The evaporated Tetrahydrofurane, Fluka, volume was compensated with the addition of deionized water to the suspension for a day or two to finally replace Tetrahydrofurane, Fluka. Thereafter, the silver nanoparticles suspension was filtered through varying pore sizes of polycarbonated isopore filters to yield different sizes of silver nanoparticles.

For the 14-day study, the different sizes of silver nanoparticles in diameters were 22 nm, 42 nm, 71 nm, and 323 nm, which were administered orally in a single dose of 1.0 mg/kg/day. For the 28-day repeated-dose toxicity study, only the 42 nm size silver nanoparticle was administered orally at 3 different doses, which were 0.25 mg/kg/day, 0.5 mg/kg/day, and 1.0 mg/kg/day. The control group was prepared with the deionized water.

Results

Homogeneity (% coefficient of variation)

Not provided

Stability (range of % of initial concentration)

Not provided

Concentration (range of % of nominal concentration)

Not provided

5. Statistics - The results of the treated group were compared using the student's t-test and one way ANOVA analysis with the control group for analyzing several parameters like accumulation of silver in the organs, serum level of TGF- β , ratio of organ to body weight exposed to varying size of silver nanoparticles, and blood biochemical parameters.

C. METHODS:

1. Observations:

1a. Cageside Observations – No specific data or information was provided on the frequency of mortality and toxicity.

1b. Clinical Examinations – No specific data or information was provided on the frequency of clinical examinations

1c. Neurological Evaluations – No neurological evaluations were conducted for this study.

Silver nanoparticles/ (PC Code Not Provided)

28-Day Oral Toxicity in (Mice) (2010) / Page 6 of 14
OPPTS 870.3050 / OECD 407

2. **Body weight:** Body weight was measured on day 0, 7, 14, 21, and 28.

3. **Food consumption:** No information or data were provided on food consumption.

4. **Ophthalmoscopic examination:** Eyes were not examined in this study.

5. **Hematology & Clinical Chemistry:** No specific data or information was provided on hematology; however, for biochemical analysis, 1 ml of blood was collected from the retro-orbital venous plexus from each mouse using heparinized capillary tubes. From the withdrawn 1 ml blood, 100 µl was used for cell phenotype analysis, and the rest was centrifuged at 3000 rpm for 10 min for preparing the serum. The serum was stored in the -80 °C freezer prior to blood biochemical analyses, which were measured using an auto-analyzer (Hitachi 7180, Hitachi, Japan). In the repeated oral toxicity test, blood was obtained from 3 mice of each group for cell phenotype and blood biochemistry analysis, and the other 3 were used for blood withdrawing for cytokine assay.

a. **Clinical Chemistry**

	ELECTROLYTES		OTHER
	Calcium	X	Albumin*
	Chloride	X	Creatinine*
	Magnesium	X	Urea nitrogen*
	Phosphorus		Total Cholesterol*
	Potassium* (K)		Globulins
	Sodium* (NA)		Glucose*
	ENZYMES (more than 2 hepatic enzymes, eg., *)		Total bilirubin
X	Alkaline phosphatase (AP)*	X	Total protein (TP)*
	Cholinesterase (ChE)		Triglycerides
	Creatine phosphokinase		Serum protein electrophoresis
	Lactic acid dehydrogenase (LDH)		
X	Alanine aminotransferase (ALT/also SGPT)*		
X	Aspartate aminotransferase (AST/also SGOT)*		
	Gamma glutamyl transferase (GGT)*		
	Glutamate dehydrogenase		
	Sorbitol dehydrogenase*		

* Recommended for 28-day oral rodent studies based on Guideline 870.305

6. **Urinalysis:** Although optional for 28-day repeated dose toxicity study, no information or data was provided on urinalysis.

7. **Sacrifice and Pathology:** Although the author did not specifically state about the histological techniques used in this study, the kidneys, livers, and small intestines of the control and treated groups were fixed with 10% neutral buffered formalin and processed using routine histological techniques. After the paraffin embedding, which was not explained in the article, 3 µm sections were cut and stained with hematoxylin and eosin for histopathologic evaluation. Injuries, cellular changes, and inflammations were examined microscopically.

Silver nanoparticles/ (PC Code Not Provided)

28-Day Oral Toxicity in (Mice) (2010) / Page 7 of 14
OPPTS 870.3050 / OECD 407

All animals after blood drawing were sacrificed and subjected to gross pathological examination check. The CHECKED (X) tissues were collected for histological examination.

	DIGESTIVE SYSTEM		CARDIOVASC./HEMAT.		NEUROLOGIC
	Tongue		Aorta, thoracic*	X	Brain*+
	Salivary glands*		Heart**		Peripheral nerve*
	Esophagus*		Bone marrow*		Spinal cord (3 levels)*
	Stomach*		Lymph nodes*		Pituitary*
X	Duodenum*		Spleen*+		Eyes (optic nerve)*
X	Jejunum*		Thymus*+		
X	Ileum*				GLANDULAR
	Cecum*		UROGENITAL		Adrenal gland*+
	Colon*	X	Kidneys**		Lacrimal gland
	Rectum*		Urinary bladder*		Parathyroid*
X	Liver**	X	Testes**		Thyroid*
	Gall bladder* (not rat)		Epididymides**		OTHER
	Bile duct* (rat)		Prostate*		Bone (sternum and/or femur)
	Pancreas*		Seminal vesicles*		Skeletal muscle
	RESPIRATORY		Ovaries*+		Skin* (treated & untreated areas)
	Trachea*		Uterus*+		All gross lesions and masses*
X	Lung*		Mammary gland*		
	Nose*				
	Pharynx*				
	Larynx*				

* Recommended for 28-day oral rodent studies based on Guideline 870.3050

+ Organ weights required for rodent studies.

8. Inflammatory responses: The author further evaluated the inflammatory responses by measuring the amount of cytokine and IgE production, and the ratio between CD4+ to CD8+ cells.

a. Measurement of cytokines – Enzyme-Linked Immunosorbent Assay (ELISA) kit was used for determining the concentrations of each cytokine in the serum. About 100 µl of capture antibody was used for coating each well of a 96-well plate and incubated overnight at 4 °C. The serum or standard was then added to each individual well after washing and blocking the plate with the assay dilution and maintained at room temperature for 2 hours. After another wash of the plate, a biotin-conjugate detecting mouse antibody was added to each well and incubated at room temperature for 1 hour. In addition, before detecting with TMB solution, the plate was washed again and further incubated with avidin-HRP for 30 min. By adding 1 M H₃PO₄ reactions were stopped, and at 450 nm the absorbance was measured with an ELISA reader. Finally, the cytokine was evaluated from the linear portion of the generated standard curve.

b. Measurement of Immunoglobulin E (IgE) – Similar to the measurement of the cytokines, the ELISA kit was used for determining the concentration of IgE in the serum. Each of the 96 well plates were coated with the coating antibody, incubated overnight at 4 °C, then washed

Silver nanoparticles/ (PC Code Not Provided)

and blocked for 1 hour at room temperature. The serum and IgE standard were added to all the wells and left for an hour at room temperature. Following another wash of the plate, detection antibodies were added to each well and incubated for an hour at room temperature. Finally with another wash the plate was further incubated with color development solution, and the reaction was terminated by the addition of 2M H₂SO₄. The absorbance was measured with an ELISA reader at 450 nm and IgE was calculated from the linear portion of the standard curve.

c. Immunophenotyping – All the T cells, B cells, NK cells, CD4+ T cells, and CD8+ T cells were identified using conjugated anti-mouse antibodies. To reduce the non-specific binding of the antibodies, blood lymphocytes were blocked with Fc-block. In addition, cells were incubated in the dark with 10 µl of the appropriate fluoro-chrome-conjugated antibody for 20 min at 4 °C, and were washed with a 500 µl Fluorescence Activated Cell Sorter (FACS). The blood was lysed and rewashed with FACS at room temperature. Thereafter, each of the samples was fixed with 1% paraformaldehyde until further analysis. The flow cytometry analysis was performed on the FACSCalibur system and matched for each fluoro-chrome with the control samples, and the data were analyzed using cellQuest software.

II. RESULTS

A. OBSERVATIONS:

1. Clinical signs of toxicity – No cageside observations and clinical examinations were provided for this study.
2. Mortality – No mortalities were reported in this study.
3. Neurological Evaluations – No neurological evaluations were conducted in this study.

B. BODY WEIGHT AND WEIGHT GAIN: Body weight changes were not provided for the 28-day study period with varying doses of 42 nm size of silver nanoparticles. However, the body weight change provided for the 14-day study with varying sizes of silver nanoparticles, did not indicate any significant changes. Overall, the average body weight did increase by 5-6 gm both in controls and the treated groups during the 14-day study to 35.15 g.

C. FOOD CONSUMPTION AND EFFICIENCY:

1. Food consumption – No information or data was provided on food consumption.
2. Food efficiency – No information or data was provided on food consumption.

D. OPHTHALMOSCOPIC EXAMINATION: No ophthalmoscopic examinations were conducted during the study.

Silver nanoparticles/ (PC Code Not Provided)

E. BLOOD ANALYSES:**1. Hematology** – No information or data was provided on hematology**2. Clinical Chemistry** – In the 28-day repeated dose toxicity test, the levels of aspartate transaminase and alkaline phosphatase were statistically significantly higher in males dosed at 1.0 mg/kg/day when compared with the controls. In females, aspartate transaminase, alanine transaminase, and alkaline phosphatase were significantly ($p < 0.01$) higher than controls.

Table 3. Clinical Chemistry of a 28-Day Oral Repeated-dose Study in Mice			
	Levels (units)	At dose level of 1.0 mg/kg/day	Control
Males	AST (IU/l)	157.33 \pm 47.98 [*]	79.00 \pm 14.18
	ALT (IU/l)	103.67 \pm 77.31	48.00 \pm 4.58
	ALP (IU/l)	124.33 \pm 37.98 ^{**}	80.67 \pm 3.79
Females	AST (IU/l)	211.67 \pm 57.18 ^{**}	79.67 \pm 5.69
	ALT (IU/l)	150.67 \pm 13.05 ^{**}	36.33 \pm 5.13
	ALP (IU/l)	181.00 \pm 51.51 ^{**}	87.67 \pm 23.46

^a Not detected.Significantly different from control group: ^{*} $p < 0.05$; ^{**} $p < 0.01$ ($n=3$, mean \pm SD).**F. URINALYSIS:** Urinalysis was not conducted.**G. Sacrifice and Pathology:****1. Organ weight** – No information was provided for the 28-day repeated toxicity study. In the 14-day study, the author stated that the organ weights of liver, kidney, testes, brain, and lungs were not affected in the treated groups. However, although it was not statistically significant, the liver and testes weights were higher than controls in mice administered 22 nm (and 42 nm for liver weight) silver nanoparticles, as the Fig.1 showed on the ratio of organ/body weight.

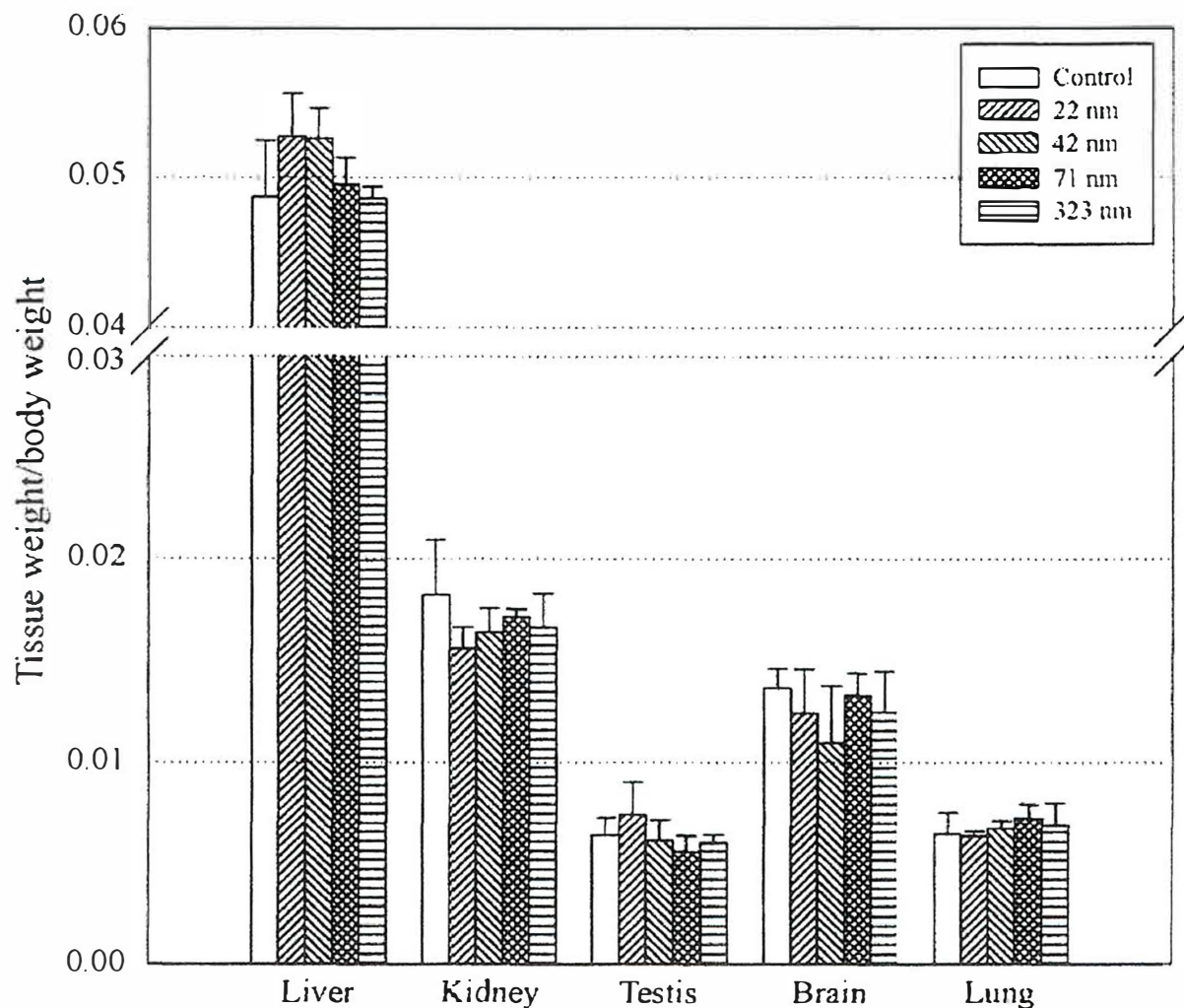


Fig 1: The ratio of organ/body weight after administration of different sized silver nanoparticles in a 14-day oral toxicity study.

2. Gross pathology – The brain seemed to be the major site of silver deposition when the mice were orally exposed to 22 nm, 42 nm, and 71 nm diameter size silver nanoparticles. A significant ($p < 0.01$) amount of silver was present in all the tested organs: brain, lungs, liver, kidneys, and testes when exposed to 22 nm. At 42 nm size silver nanoparticles, lungs, kidneys, and testes had significant ($p < 0.01$) silver accumulation, and the liver and kidneys at 71 nm silver nanoparticles size had significant ($p < 0.01$) amount of silver, when compared to controls. However, at 323 nm size of silver nanoparticles, no deposition was observed in any of the organs tested (see Table 4 below).

The organs in the 28-day repeated oral toxicity study were not tested for silver accumulation.

Silver nanoparticles/ (PC Code Not Provided)

Table 4. Accumulation of Silver in the Organs in a 14-day Oral Study of Various Sized Nanosilver Particles in Mice at a Single Dose of 1.0 mg/kg/day					
Organs	22 nm	42 nm	71 nm	323 nm	Control
Brain	255.56 ± 3.13**	138.80 ± 1.85**	51.32 ± 3.56**	ND ^a	ND
Lung	208.11 ± 7.65**	171.11 ± 9.05**	13.00 ± 22.50	ND	ND
Liver	265.41 ± 1.55**	86.67 ± 150.11	220.95 ± 7.48**	ND	ND
Kidney	345.76 ± 10.75**	47.92 ± 16.40**	53.21 ± 1.53**	ND	ND
Testis	316.00 ± 5.40**	172.63 ± 8.75**	ND	ND	ND

^a Not detected.

Significantly different from control group: ** p<0.01 (n=5, mean ± SD).

3. Microscopic pathology – In the 28-day repeated-dose study, oral administration of silver nanoparticles at 1.0 mg/kg/day, a slight cell infiltration was observed in the kidney's cortex region in males and females. No other significant histopathological changes were reported in the treated groups of the 28-day study.

4. Inflammatory responses – In order to assess the inflammatory responses, the concentration of pro-inflammatory cytokines (IL-1, IL-6, TNF-α), Th-1 type cytokines (IL-12 and IFN-γ), Th-2 type cytokines (IL-4, IL-5, IL-10) and TGF-β in serum, and IgE were measured. In the 28-day repeated-dose toxicity study, an increase in all the above indicators was observed in mice treated with 1.0 mg/kg/day silver nanoparticles when compared with controls. In the 14-day study, the level of TGF-β in serum was significantly higher in mice treated with 22 nm, 42 nm, and 71 nm silver nanoparticles.

Table 5. Inflammatory Responses a 28-Day Oral Repeated-dose Study in Mice			
	Levels (units)	At dose level of 1.0 mg/kg/day	Control
Pro-inflammatory cytokines	IL-1 (pg/ml)	8.8 ± 07.0**	ND ^a
	IL-6 (pg/ml)	13.75 ± 0.57**	1.44
	TNF-α (pg/ml)	3.41 ± 0.06	1.21
Th-1 type cytokines	IL-12 (pg/ml)	76.86 ± 5.20**	35.5
	IFN-γ (pg/ml)	0.52 ± 0.04	ND
Th-2 type cytokines	IL-4 (pg/ml)	2.7 ± 0.07*	ND
	IL-5 (pg/ml)	1.34 ± 0.01	ND
	IL-10 (pg/ml)	29.02 ± 1.70**	ND
TGF-β	(pg/ml)	6.73 ± 0.52**	ND
IgE	(ng/ml)	6.04 ± 0.74*	3.28

^a Not detected.

Significantly different from control group: * p<0.05; ** p<0.01 (n=3, mean ± SD).

In addition, not significant but an increase in distribution of NK cells and B cells were reported in both 14-day study with 22 nm silver nanoparticles and 28-day study at 1.0 mg/kg/day dose level. When compared to controls with the CD4+/CD8+ ratio of 3.99 in the 14-day study, the lowest CD4+/CD8+ ratio of 2.88 was reported with exposure to 22 nm size silver nanoparticles among all the other silver nanoparticles tested. Likewise, in the 28-day study, at 1.0 mg/kg/day dose, the CD4+/CD8+ ratio was 2.76 versus 3.80 of controls.

III. INVESTIGATORS' DISCUSSION AND CONCLUSIONS: In the 14-day study, the author described that the oral administration of 22 nm size silver nanoparticles were easily translocated into the blood stream and accumulated significantly ($p < 0.01$) within the organs. The brain and kidneys had significant amount of silver nanoparticles deposition when exposed to 22 nm, 42, and 71 nm silver nanoparticles. The authors indicated the size dependent penetration along with the brain, in the lungs and testes.

In the 28-day repeated-dose toxicity test, statistically significant increases in liver enzymes (aspartate transaminase, alkaline phosphatase, and alanine transaminase) were reported at 1.0 mg/kg/day dose level. The author indicated that this increase was solely due to the silver nanoparticles administered without any vehicle. In addition, the author suggested that since the study did not find any histopathological changes in the liver despite the increase in the enzymes secretion, further studies are required on the same. Also, the concentration of pro-inflammatory cytokines (IL-1, IL-6, TNF- α), Th-1 type cytokines (IL-12 and IFN- γ), Th-2 type cytokines (IL-4, IL-5, IL-10), TGF- β in serum, IgE, B-cell and CD8+ distribution were higher in the 28-day repeated-dose study at 1.0 mg/kg/day dose compared to controls. **The author did not determine any NOAEL or LOAEL values for either the 14-day or 28-day repeated-dose toxicity study.**

A. REVIEWER COMMENTS: The agency agrees that the size of the silver nanoparticles along with the route of exposure renders its translocation into the blood stream and deposition within the organs. In this case, the smallest, 22 nm diameter size silver nanoparticles demonstrated the greatest accumulation in all the tested organs: the brain, lungs, kidneys, testes, and liver compared to the other sizes tested, and the accumulation was significantly ($p < 0.01$) higher than controls. The largest silver nanoparticle tested was 323 nm in diameter, which did not accumulate in any of the tested organs. All the relatively smaller sizes (22, 42, 71 nm) to 323 nm had significantly ($p < 0.01$) accumulated in the brain and kidneys, but the accumulation in the brain reduced with increase in silver nanoparticles size. This penetration as indicated by the author raises concerns about the retention and elimination of the nanoparticles after it has accumulated within the body. Additional investigations must adhere to understanding the biotransformation of silver nanoparticles at small sizes and low-doses. Also, clarification on observing a sudden decrease in silver nanoparticles accumulation in the liver and kidneys at 42 nm must be provided.

In the 28-day repeated-dose toxicity study, as suggested by the author, further analysis of the liver is required to comprehend the statistically significant increase in the liver enzyme despite not observing any histopathological changes/damages to the liver. Due to the lack of data on the

Silver nanoparticles/ (PC Code Not Provided)

significant levels of the increase in cytokines (Th-1 type cytokines and Th-2 type cytokines), B-cells, and allergic response (IgE), the author is required to provide additional data on the same.

The reduced ratio of CD4+/CD8+ in both the 14-day and 28-day repeated dose toxicity study as mentioned by the author is an "increase in the CD8+ cell distribution". This increase in the CD8+ T cells means that the suppressor-cytotoxic T cells are more in numbers, which indirectly signifies that the silver nanoparticles affected the CD4+ T cells by reducing their numbers. Thus, the increase in CD8+ T cell distribution as specified by the author is due to what? Moreover, for the production of CD8+T cells, which are predominantly activated by a set of reactions from the CD4+ T cells, specifically T-helper cells are necessary. Thus, the author must provide substantial reasoning to explicitly describe this increase in CD8+ cell distribution in the study by either providing an initial count of both CD4+ and CD8+ cells before commencing the experiment on their ratio to lucidly understand the actual effect of silver nanoparticles on the same.

For the 28-day repeated dose toxicity study in mice, with 42 nm size silver nanoparticles, the NOAEL = 0.50 mg/kg/day. The established LOAEL = 1.0 mg/kg/day, based on the statistically significant changes in clinical chemistry including increase in serum liver enzymes of the aspartate transaminase, alkaline phosphatase in males, and aspartate transaminase, alanine transaminase, and alkaline phosphatase in females. Other factors can be considered for supporting the LOAEL value pending the receipt of clarification from the author on the statistical significance level of cytokine production, B-cell distribution, and inflammatory cell infiltrates in kidney at 1.0 mg/kg/day dose level.

B. STUDY DEFICIENCIES:

Major deficiencies for the 28-day repeated-dose toxicity test:

1. The purity of silver nanoparticles was not provided
2. Additional data on statistical significance level of cytokine production, B-cell distribution, and inflammatory cell infiltrates in kidney at 1.0 mg/kg/day dose level.
3. No information on neurological evaluations and hematology were provided
4. Increase in cell distribution of CD8+ as stated by the author from analyzing the CD4+/CD8+ ratio in 28-day repeated-dose study is required.
5. Several other organs that are mandatory for sacrifice and pathology must be studied for this 28-day repeated-dose study.
6. Data must be provided on urinalysis, hematology, and detailed gross necropsy.
7. Need for studying the inflammatory responses in mice in this study was not elucidated in the introduction section of the article. The author must provide supporting evidence on the same.

Minor deficiencies:

8. Information or data is required on test substance homogeneity, food consumption, food efficiency, ophthalmological examination, cage-side observation, and mortalities.

C. STUDY CLASSIFICATION: This 28-day repeated-dose toxicity study in mice is **unacceptable/upgradeable-guideline** because it does not satisfy the guideline requirement for a 28-day oral rodent study based on Guideline 870.3050, but it's upgradeable if information on the purity of silver nanoparticles, data on statistical significance on cytokine production, B-cell distribution, inflammatory cell infiltrates are provided. Also, reasoning for observing an increase in the CD8+ cell distribution while analyzing the CD4+/CD8+ ratio must be adequately addressed. Others, including food and water consumptions, neurological evaluations, hematology and ophthalmological examinations, and clinical biochemistry determinations along with a detailed gross necropsy, are also required.

Sign-off Date : 03/29/11

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